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Residual Dipolar Coupling Derived Orientational Constraints on Ligand Geometry in a 53 kDa Protein-Ligand Complex

P. J. Bolon, H. M. Al-Hashimi and J. H. Prestegard*

Complex Carbohydrate Research Center, University of Georgia Athens, GA 30602, USA

**Corresponding author*

The geometric relationships between ligands and the functional groups that bind ligands in soluble ligand-protein complexes have traditionally been deduced from distance constraints between pairs of NMR active nuclei spanning the ligand-protein interface. Frequently, the steep inverse distance dependence of the nuclear Overhauser effect (NOE), from which the distance constraints are derived, makes identification of sufficient numbers of constraints difficult. In these cases the ability to supplement NOE-derived information with distance-independent angular information can be very important. Here, the observation of residual dipolar couplings from α-methyl mannose bound to mannose binding-protein in a dilute liquid crystalline medium has allowed the determination of a bound ligand's average orientation. The 3-fold rotational symmetry of mannose-binding protein defines its orientational tensor and obviates the need to determine experimentally the protein's average orientation. Through superimposition of ligand and protein orientational tensors we describe the binding geometry of α-methyl mannose bound to mannose-binding protein. This new method is of general applicability to the study of ligands bound to proteins, and it is of particular interest when neither X-ray crystallography nor NOE techniques can provide sufficient information to describe binding geometries.

Keywords: dipolar couplings; NMR; structure determination; carbohydrate interactions; mannose-binding protein

Introduction

We illustrate here a new approach for the determination of ligand geometry in protein binding sites that relies on orientational constraints derived from residual dipolar couplings observed in NMR spectra of partially ordered macromolecular complexes (Bothner-By, 1995; Prestegard, 1998; Prestegard *et al.*, 1998; Tjandra & Bax, 1997; Tolman *et al.*, 1995). The determination of the bound geometry of protein ligands is an important issue for improving our understanding of how structure relates to biological function, and also for building a basis for rational drug design. Many useful approaches to this determination have been developed in the past, including X-ray diffraction of crystalline protein-ligand complexes and NMR-based transfer NOE studies of complexes in solution (Ni, 1994). All the approaches are limited in certain ways. In the case of X-ray crystallography, complexes must be crystallizable; in the case of transferred nuclear Overhauser effect (NOE) studies, ligands must be in rapid exchange. Transfer NOE studies, while giving sound information on ligand geometry, also suffer from a lack of information on the nature of ligand protein contacts. Information on these contacts can be provided by more conventional NOE studies using both protein and ligand resonances in some cases, but this information is often difficult to obtain because it requires assignment of protein as well as ligand resonances, and because it presumes sufficiently close approach of protein and ligand protons to allow efficient magnetization transfer ($r < 5$ Å).

Cases where determination of bound ligand geometry have been particularly difficult include those involving protein-oligosaccharide interactions.

Abbreviations used: NOE, nuclear Overhauser effect; MBP, mannose-binding protein; CRD, carbohydrate recognition domain; AMM, α-methyl mannoside; HSQC, heteronuclear single quantum coherence.

E-mail address of the corresponding author: jpresteg@ccrc.uga.edu

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These cases encompass an important area in that protein-oligosaccharide interactions mediate a variety of processes essential to cell function and survival. To name a few, sialyl Lewis-X oligosaccharide-selectin interactions mediate the recruitment of leucocytes to sites of injury. The interaction of choleratoxin with the oligosaccharide headgroup of ganglioside GM_1 mediates internalization of the toxic subunit, and polylactosamine-galectin interactions are hypothesized to be involved in the metastasis of malignant cells (Lasky, 1992). Characterization by traditional NMR methods of the oligosaccharide-protein interactions that mediate these processes is difficult because the hydrogen-bonding networks involving hydroxyl protons on the sugars are often part of the interface between protein and oligosaccharide. The hydroxyl protons are then the ones in closest proximity to protein protons, but these exchange rapidly with protons in bulk water making their NMR resonances hard to observe. In these difficult cases, residual dipolar data can offer a valuable alternative, where the data can in principle constrain both bound ligand geometry and ligand orientation relative to the protein binding site.

Our particular target in the studies presented here is mannose-binding protein-A (MBP). MBP is a protein important in innate immune response. It binds sugars, including α-mannosides, that are frequently exposed on the surface of invading pathogens, and subsequently triggers a complement cascade (Ikeda *et al.*, 1987). Improper activation *via* MBP has also been suggested as a factor in inflammatory disease (Dwek, 1996). Crystal structures of several forms of MBP are available. The form we study here is a 149 residue truncated version that includes a calcium-containing carbohydrate recognition domain (CRD) and a short α-helical stem that initiates the formation of a homo-trimer by making a left-handed coiled-coil with helical stems from two other MBP monomers. The 3-fold symmetry axis in the resulting homo-trimeric structure is important for the interpretation of our results. The binding of several ligands to MBP and mutant MBPs that mimic selectin proteins has also been studied, both because of interest in MBP's ligand binding properties and those of proteins which show homology to the MBP CRD, such as the selectins. Crystallography has been the primary contributor in studies of MBP trimers, with structures existing for bound galactoside and galactosamine, as well as sialyl Lewis-X and related ligands (Chang *et al.*, 1994; Iobst *et al.*, 1994; Kolatkar AR, 1998; Ng & Weis, 1997; Weis & Drickamer, 1994). Additionally, there are structures of a shorter fragment of MBP which makes a homo-dimer; one of these includes α-methyl mannoside in the carbohydrate binding site (Ng *et al.*, 1996). The results of the above crystallography studies provide a useful basis for evaluating data from our new approach.

The new approach that we present is based on the observation of residual dipolar coupling of directly bonded ^1H -^{13}C pairs in α-methyl mannoside (AMM) when the mannoside is bound to MBP in a field-oriented aqueous liquid crystal. The use of residual dipolar coupling in the characterization of the structures of proteins and other macromolecules in solution has received a good deal of attention recently (Clore *et al.*, 1998a; Tjandra & Bax, 1997) . When molecules are partially ordered in a magnetic field interaction, vectors connecting magnetic nuclei in the molecules depart from a complete isotropic sampling of all directions in space. This gives rise to a contribution to splitting of NMR resonances that depends on the magnetic properties of the interacting nuclei, the internuclear separation (r) and the angle (θ) that the vector makes with the magnetic field. When the nuclei are directly bonded as in a ^1H-^{15}N or ^1H-^{13}C pairs, the $1/r^3$ distance-dependence of the interaction can be regarded as being determined by the bond length and the primary variable becomes the orientation of the bond relative to the magnetic field as characterized by the angle θ. The functional dependence of the dipolar couplings is $(1/2(3\cos^2\theta - 1))$, where the parentheses denote an average over an orientation distribution, and the resulting interaction appears as an addition to the normal one bond scalar couplings. These through space interactions are always present; in fact, they are the basis of the common NOE interaction. However, the angular function above is averaged to zero when space is isotropically sampled and direct splittings of resonances only appear in partially oriented media. Fortunately, several means of producing field-ordered states are now available, including inherent orientation due to the large anisotropies in the magnetic susceptibilities of some molecules (Kung *et al.*, 1995; Tolman *et al.*, 1995), orientation due to the interaction of molecules with lipid bicelles that form field-oriented liquid crystals (Tjandra & Bax, 1997) and interaction with filamentous bacteriophage that also form field-oriented liquid crystals (Clore *et al.*, 1998c; Hansen *et al.*, 1998).

The interpretation of residual dipolar data has taken two routes, incorporation of individual bond constraints as penalty functions in simulated annealing protocols for molecular structure determination (Clore *et al.*, 1998a,b), and extraction of order tensors that directly describe the direction and level of ordering forces from the point of view of a coordinate frame fixed in a rigid molecular fragment (Losonczi *et al.*, 1999; Losonczi & Prestegard, 1998b; Saupe, 1968). The latter approach which arose from substantial previous work in the liquid crystal field, proves to be particularly valuable for the problem at hand. An order tensor is a 3×3 matrix with elements $(1/2(3\cos\theta_i\cos\theta_j - \delta_{ij}))$ written in terms of direction cosines that relate Cartesian axes of an arbitrarily chosen molecular axis system to the magnetic field direction. Because the matrix is traceless and symmetric, there are only five independent elements. An order matrix in an arbitrarily chosen frame of a molecular fragment is difficult to inter-

pret, but if diagonalized, the five independent variables turn into a principal order parameter, an asymmetry parameter and three Euler angles relating the diagonal, or principal frame, to the original molecular frame. If fragments of interest experience a common source of order, as they would if they were parts of a rigid ligand-protein complex, the directions and levels of the orienting force should appear the same from the point of view of each fragment when a model for the fragments is assembled with proper fragment orientations. This provides a test for the validity of proposed models for the orientation of a ligand (one fragment) in a protein binding site (another fragment).

The key to using this test is having an approximately rigid fragment with enough potential dipolar coupled pairs to determine the five elements of an order tensor. For a ^{15}N-labeled protein, pairs of ^{1}H-^{15}N spins are clearly abundant and much of the protein backbone can be considered rigid. In oligosaccharides, individual pyranose rings frequently prefer a $^{4}C_1$ configuration which can be considered to have approximately rigid geometry and ^{1}H-^{13}C pairs are abundant. In α-methyl mannoside there are five directly bonded ^{1}H-^{13}C pairs on the $^{4}C_1$ ring, two of which point in two unique directions and three of which depart slightly from a third direction. These pairs can provide the data for an order tensor determination. There are a number of ways of accurately determining the splittings of resonances from these pairs (Tolman & Prestegard, 1996) but the easiest is simply to collect ^{1}H-^{13}C heteronuclear single quantum coherence (HSQC) spectra, without decoupling in one of the frequency domains. This is the approach we use here. We used uniformly ^{13}C-labeled α-methyl mannoside in order to improve sensitivity.

Results and Discussion

Measurement of residual dipolar couplings in AMM complexed to MBP

The ^{1}H-^{13}C HSQC NMR spectra of ^{13}C-enriched AMM in the presence of an equimolar amount of MBP dissolved in a dilute bicelle medium are shown in Figures 1(a) and (b) at temperatures of 25 °C and 39 °C, respectively. The data were acquired in the absence of ^{13}C decoupling during acquisition, and the one bond ^{13}C-^{1}H couplings are shown as frequency domain splittings in the proton dimension. The magnitudes of these couplings and the corresponding errors extracted using a Bayesian parameter estimation method (Andrec & Prestegard, 1998) are shown on the spectra. As one would expect for the case of isotropic tumbling at 25 °C, all the couplings in Figure 1(a) are similar and fall in the range of typical $^{1}J_{CH}$ scalar coupling constants for non-anomeric sites in carbohydrates (140-150 Hz). On the other hand, couplings measured at 39 °C are more widely spread because of the presence of a residual dipolar contribution. Residual dipolar contributions for individual C-H bond vectors calculated from the differences in couplings measured at 25 °C (isotropic) and 39 °C (aligned) are shown in Table 1 (D_{obs}).

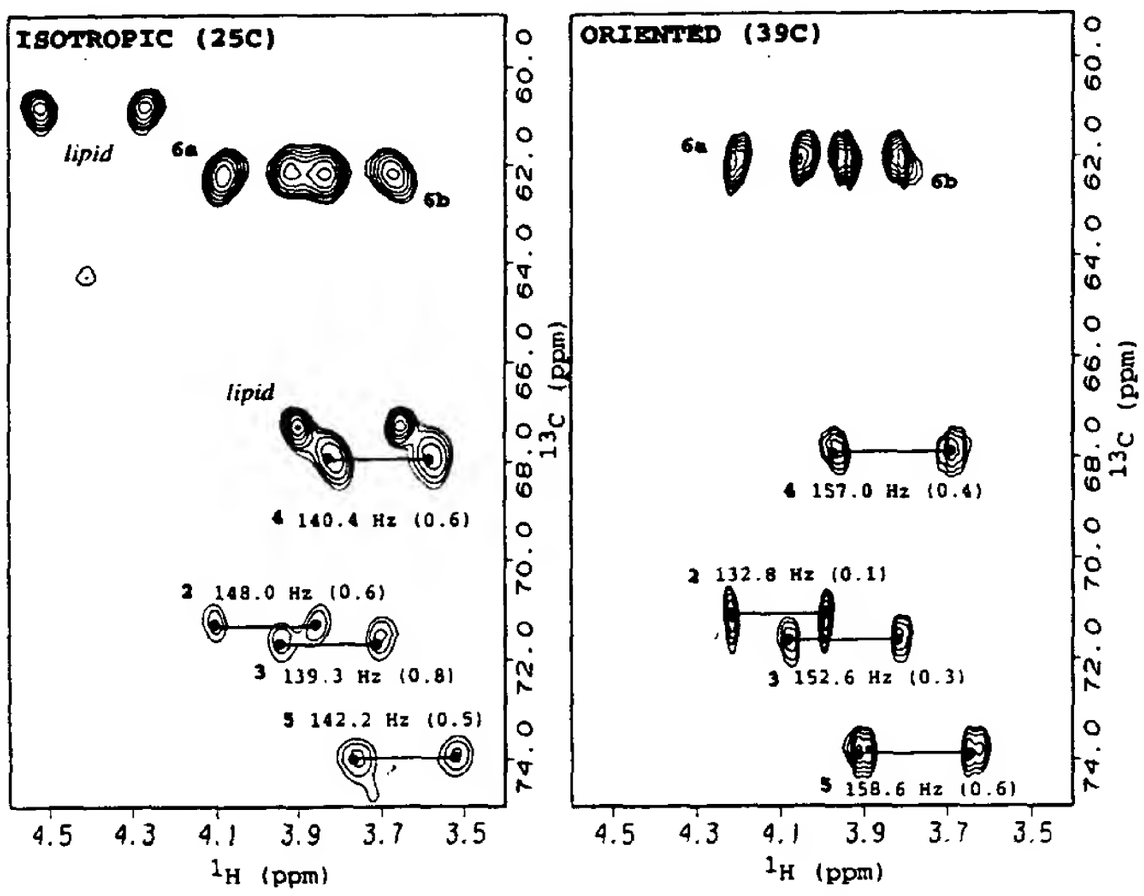

Figure 1. (a) Section of a proton-coupled HSQC spectrum of isotropic AMM in MBP with selected couplings annotated. (b) Section of a proton-coupled HSQC spectrum of oriented AMM in MBP with selected couplings annotated. The anomeric regions of the spectra are not shown but show splittings of 169.4(±0.5) Hz for isotropic condition and 158.6(±0.2) Hz for the oriented case.

Table 1. Residual dipolar couplings for bound and free AMM

Data Set	C_1-H_1 (Hz)	C_2-H_2 (Hz)	C_3-H_3 (Hz)	C_4-H_4 (Hz)	C_5-H_5 (Hz)
AMM + MBP + bicelle (D_{obs})	−10.8±0.7	−15.2±0.7	13.3±1.2	16.6±1	16.4±1.1
AMM + bicelle (D_{free})	−3.5±0.7	−7.3±0.7	9.2±0.9	12.1±0.7	7.8±0.5
AMM-bound state (D_{bound})	−22.6±2.2	−28.7±2.2	20±3.5	23.9±2.9	30.5±3.0

While residual dipolar coupling contributions are indeed observed in the AMM/MBP complex dissolved in a bicelle medium, they need not originate fully from binding of AMM to a strongly aligned MBP molecule. We see only a single set of resonances, but for a weak binding ligand in fast exchange that spends a portion of its time bound to the protein and the remainder free in solution, this will always be the case. Thus, observed splittings will be a population weighted average of those induced in the free and bound states. Residual dipolar couplings have previously been observed in free carbohydrate molecules dissolved in bicelle media (Bolon & Prestegard, 1998; Kiddle & Homans, 1998; Rundlof *et al.*, 1998). While small, they can be significant. In order to quantify the contribution from the AMM-free state, the same experiments were repeated under identical conditions in the absence of MBP. The differences in measured couplings between 25 °C and 39 °C are also shown in Table 1 (D_{free}). Again, couplings and associated errors were extracted using a Bayesian parameter estimation method. It is noteworthy that the magnitudes of couplings for various C-H bond vectors are generally smaller for free AMM samples, but the reduction varies from site to site. The different pattern of splittings assures us that we are not looking at a simple scaling of bicelle-induced order and that MBP has a significant direct effect on both extent and direction of AMM alignment. This direct binding effect was confirmed in another set of experiments. Here, a molar ratio of 1:8 MBP:AMM was used, which would reduce the bound AMM state to approximately 8%. Indeed, in this case where indirect effects would dominate, the presence of MBP had little measurable effect on the observed residual dipolar couplings.

Interpretation of residual dipolar contributions of a ligand in a protein-bound state will, in general, require separation of the contribution from the free state using known binding properties. The observed residual dipolar couplings measured in the AMM/MBP complex (Table 1, D_{obs}) will be a population weighted average from the free (N_{free}) and bound (N_{bound}) states, such that:

$$D_{obs} = N_{free}D_{free} + N_{bound}D_{bound} \quad (1)$$

The fraction of AMM free and bound (N_{free} and N_{bound}) can be calculated from the dissociation constant and known protein-ligand concentrations. Using a previously determined binding constant of ≈ 1 mM (Sayers, 1998), approximately 40% of AMM is in fact bound to MBP leaving approximately 60% of AMM free in solution. Residual dipolar couplings originating from the bound state (D_{bound}) calculated from equation (1) are shown in Table 1. Since these dipolar contributions originate from MBP's partial alignment, they contain the desired orientational information on AMM relative to MBP. This information is best extracted using an order matrix analysis.

Order tensor calculation in AMM

Using AMBER-minimized structure coordinates for AMM (Woods *et al.*, 1995) and five residual dipolar couplings measured in the sugar ring in simple bicelle solution, the five elements of the order matrix were determined using a singular value decomposition approach (Losonczi *et al.*, 1999). The initial molecular coordinate frame for AMM was defined by placing the *y*-axis along the C1-H1 vector and the *z*-axis normal to the plane defined by H1-C1-OMe. Using this frame, the position of the principal order frame axes determined from residual dipolar couplings from free AMM (D_{free}, Table 1) is illustrated in Figure 2(a) using a Sauson-Flaumsteed projection (Bugayevskiy & Snyder, 1995). The alignment tensor is extremely asymmetric and the directions of all three principal axes of the alignment tensor are well defined, except that inversion of any axis is allowed. The asymmetry is quantitatively defined by an asymmetry parameter eta (η) that can be written in terms of order parameters S_{ij} ($\eta = (S_{yy} - S_{xx})/S_{zz}$). It is equal to 0.8(±0.13). Asymmetric behavior is not unusual and has, in fact, been observed in many systems dissolved in bicelle media. Due to the high level of asymmetry, the direction of highest order (S_{zz}) can alternate between two orthogonal orientations (Figure 2(a)) for which S_{zz} assumes values similar in magnitude but opposite sign ($S_{zz} = +0.0005$ or -0.0005). Note that one of the possibilities for the higher level of order is nearly along the *x*-axis of the initial molecular coordinate frame (the center of the Sauson-Flaumsteed plot). Hence, it is nearly in the plane of the H1-C1-OMe fragment and perpendicular to the C-H vector.

The five elements of the order tensor for AMM determined from couplings for the MBP-bound state (D_{bound}) were also determined using a singular value decomposition method, and the directions of the principal axes are depicted in Figure 2(b). While the position of the z-axis, the most strongly ordered axis, is well defined, the position of the S_{xx} and S_{yy} axes are ambiguous.

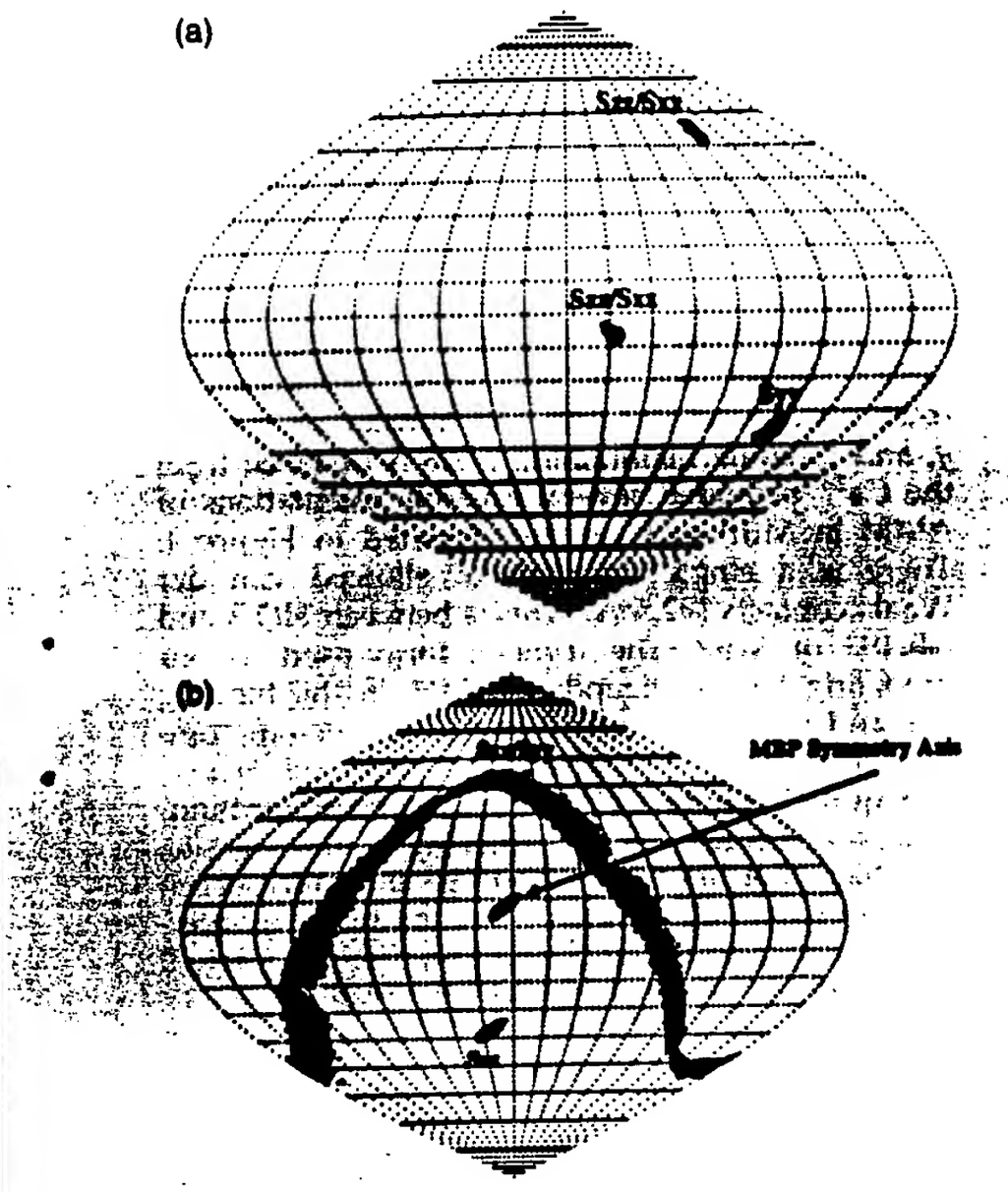

Figure 2. (a) Sauson-Flaumsteed projection of the directions of highest order for oriented AMM without MBP. (b) Sauson-Flaumsteed projection of the directions of highest order for oriented AMM in the presence of MBP, with a clear depiction of axial symmetry. The molecular coordinate frame for AMM was defined by placing the *y*-axis along the C1-H1 vector and the *z*-axis normal to the plane defined by H1-C1-OMe.

This is a characteristic of an axially symmetric order tensor. The asymmetry parameters confirms this axially symmetric nature of the alignment tensor with $\eta = 0.24(\pm 0.16)$. In addition, the level of order ($S_{zz} = -0.0012(\pm 0.00012)$) is approximately 2.5 times larger than that for AMM in free solution, and the direction of highest order is shifted 40° toward the molecular z-axis (out of the plane of to the H1-C1-OMe fragment).

The direction of alignment for the bound ligand is most useful when referenced to the alignment of MBP. We know that the direction of highest order (S_{zz}) depicted in Figure 2(b) should coincide with the direction of highest order for MBP itself. Rotating the molecular frame of the sugar to achieve coincidence then allows determination of the relative orientation of AMM bound to MBP. In general, determination of the alignment from a protein is possible using ^{1}H-^{15}N data as we have recently demonstrated for other systems (Fischer *et al.*, 1999). However, careful consideration of MBP's structure, and in particular, its symmetry, allows us t omit this independent interpretation and indeed explain the appearance of axial symmetry depicted in Figure 2(b).

MBP's alignment tensor: a solution in symmetry

The form of MBP used in these studies is a homo-trimer with a 3-fold symmetry axis and three equivalent sugar binding sites related by the same symmetry operation as depicted in Figure 3. The computed residual dipolar couplings originating from the AMM in the bound state (D_{bound}) will thus be an average over three orientations that are related by MBP's 3-fold symmetry axis. As has long been recognized and used in studies of small molecules (Saupe, 1968) this averaging has a very specific effect on order matrices determined from averaged couplings; regardless of the nature of MBP's interaction with the bicelle medium, AMM's experimentally determined direction of highest order must always point along the symmetry axis of MBP and the resulting order tensor must always be axially symmetric (Al-Hashimi *et al.*, 1999).

In the absence of any large conformational flexibility and mobility in the bound state, the order parameters determined from bound AMM ($S_{zz} = -0.0012(\pm 0.00012)$) will reflect MBP's order parameter. The fact that the principal order parameter is negative indicates that MBP's symmetry axis is, on average, perpendicular to the magnetic field and parallel with the normals of the bicelles in our

Figure 3. Depiction of AMM in fast exchange with the three equivalent CRDs of MBP. The 3-fold axial symmetry results in a direction of highest order along the rotor axis. Yellow spheres correspond to Ca^{2+}, black and red shperes to carbon and oxygen, respectively, of AMM, and MBP is represented by ribbon diagram.

medium (these order with their normals perpendicular to the magnetic field; Sanders *et al.*, 1994). This is as one would expect if interactions with the surfaces of bicelles dictated order of MBP. Axial symmetry should also propagate to the bound ligand and this is exactly what we observe in the order tensor determination using residual dipolar contributions from MBP bound AMM as shown in Figure 2(b). The direction of highest order, S_{zz}, in Figure 2(b) should now point along the symmetry axis of MBP. As we discuss below, the orientation of AMM deviates by about 40° from expected results using modeling studies and available crystal structures.

The orientation of AMM bound to MBP

No crystal structure of trimeric MBP-A with AMM in the binding site actually exists. However, AMM in the site of a dimer of the homologous MPB-C, missing the helical stem exists, and AMM in the trimer can be positioned by overlaying a carbohydrate-binding domain from the dimer on the three domains in the trimer structure (PDB entries 1KMB and 1RDL, rmsd 0.63-0.78 Å). The orientation of AMM, when positioned so that the experimentally determined symmetry axes coincide with that of MBP cannot, however, be made to overlap with the orientation of AMM modeled in this way. It is displaced by approximately 40°. There are several possible explanations for this observed deviation. First, our use of the trimer as a model may be in error. Instead, an equilibrium among monomer, dimer and trimer forms may exist in solution. This possibility is unlikely, since linewidths and diffusional properties of the construct used in previous studies are consistent with a trimer (Sayers, 1998). Moreover, the experimentally determined axially symmetric tensor is consistent with the expected 3-fold averaging. Second, the actual geometry of the trimer in solution may be different from what is depicted in the crystal structure. This is not out of the question; there are in fact X-ray structures of mutated MBPs in trimeric aggregates which show substantial differences in the relative placement of the CRDs, even though mutations are concentrated in loops at the extremities. For example, the helices near the trimer junction in PDB entries 1KMB and 1AFA are displaced from one another by more than a 1 Å when the stems are aligned. Similarly, the presence of internal motion in each protein domain or in AMM in the bound state can lead to deviations of average orientations. Third, it may be that alternate binding geometries are possible. A second binding site, involving a second Ca^{2+}, in each CRD has, in fact, been observed in crystals containing a high level of saccharide (Ng *et al.*, 1996). If this site were occupied, a proper model would have to include averaging of the orientation of the sugar in this site. Note that the second and third cases would preserve the axially symmetric nature of the determined tensor but the direction of highest order will vary. A fourth possibility is that the binding geometry in the primary site can vary.

In Figure 4 we show a geometry of AMM in the binding site of MBP (1KMB.pdb) that is consistent with our determined axis of symmetry. Using this refined structure, α-methyl mannose was docked to the binding calcium of the CRD of MBP. Orientational constraints required placing AMM's experimentally derived direction of highest order along MBP's 3-fold axis of symmetry. Subsequently, rotations around this axis and translation along x, y, and z, while maintaining a 2.6 Å distance from the Ca^{2+} provided several possible orientations of AMM in MBP such as that depicted in Figure 4. There is a single violation of allowed van der Waals contacts (<2.5 Å). This is between HO-3 and Glu193 of MBP. The trimeric form used in our modeling has no ligand of AMM in the binding site and Glu193 coordination of the calcium compensates for the absence of sugar hydroxyl. Hence, it is quite likely that Glu193 moves on sugar binding. MBP actually has a rather open binding site, in which coordination of the three and four oxygen atoms of the sugar to Ca^{2+} are the primary interactions and considerable variation in the actual geometry of coordination may be possible.

An independent determination of MBP's order tensor would remove some of the uncertainties in the above discussion. First, this would allow determination of the relative domain orientations of MBP in solution. This can be achieved by equating the observed direction of highest order as seen from any single domain to the symmetry axis of trimeric MBP (Al-Hashimi *et al.*, 1999). This procedure, in fact, introduces a novel and simple route into determination of relative domain orientations in certain types of symmetry related homo-multimers such as MBP, a problem that so far has been

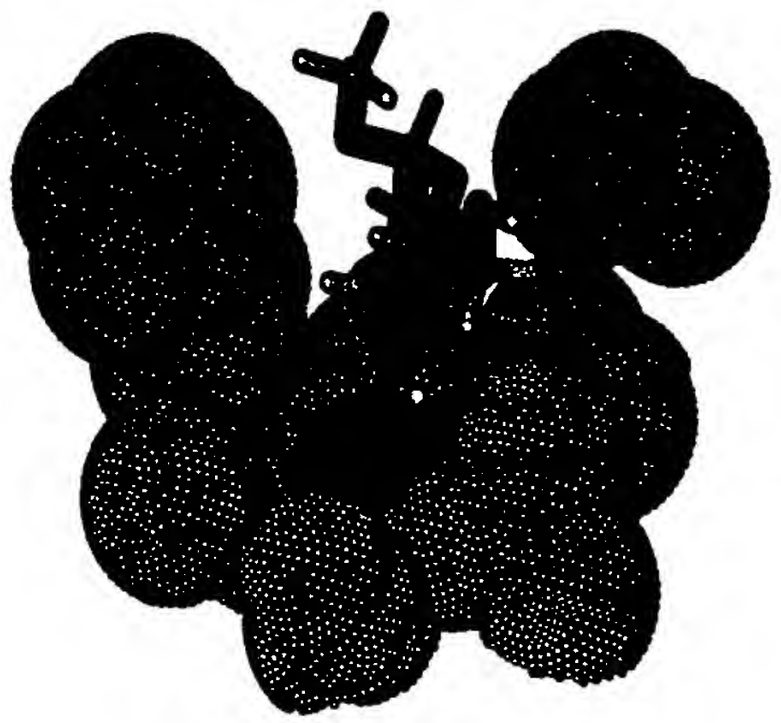

Figure 4. Proposed mode of binding of AMM in MBP. The dark blue corresponds to Ca^{2+}, and the light blue represents residues in the binding pocket of MBP.

very difficult to address using standard NMR techniques. Second, the determined direction of highest order and the corresponding order parameters will have absorbed any internal motional averaging contributions within MBP permitting direct comparison with an order tensor determined for AMM in the bound state. Additional averaging in the ligand site, either due to mobility in the bound [illegible] of alternative binding [illegible] protons on the ligand and the protein. The effects of symmetry on measured residual dipolar couplings provides a powerful orientation reference for not only ligand orientation, but possibly also for the relative orientation of domains in homo-multimeric complexes.

Materials and Methods

Preparation of MBP-A

MBP-A is an expression product from the pIN-IIIompA-2 plasmid (Drickamer, 1989), and was expressed as described (Weis *et al.*, 1991). Briefly, competent JA221 cells were transformed with DNA plasmid and colonies selected from LB-amp plates for use in a starter culture in an LB-amp medium. Starter culture was grown to saturation overnight at 37 °C and used to inoculate five liters of LB-amp medium, which grew to A_{550} of 0.8. MBP-A production was then induced with IPTG and additional growth allowed for 2.5 hours. Cells were harvested by centrifugation at 4000 *g*, the pellet was resuspended in 10 mM Tris-Cl (pH 7.0) and then lysed by sonication. The insoluble pellet from 18,000 *g* centrifugation was solubulized in 6 M guanidinium chloride, 0.1 M Tris-Cl (pH 7.0), and clarified by centrifuging at 138,000 *g* for 30 minutes at 4 °C. Dialysis of the centrifuge extract with 25 mM Tris-Cl, 1.25 M NaCl, 25 mM $CaCl_2$ over 48 hours and centrifuging at 138,000 *g* provided the dialysate which was purified by affinity chromatography on a mannose-Sepharose column. Elution with 25 mM Tris-Cl, 1.25 M NaCl and 2.5 mM Na_2EDTA followed by reconstitution by dialysis against 10 mM NaCl, 1 mM Tris-Cl, 25 mM $CaCl_2$ afforded 10 mg of MBP-A from a five liter growth culture.

Preparation of AMM

AMM was synthesized from [$^{13}C_6$] glucose by minor modification of a described method (Hare *et al.*, 1993). Briefly, uniformly ^{13}C-labeled D-glucose was protected as the penta-acetate with acetic anhydride, and the crude material brominated with HBr/AcOH. Reduction with Zn/aqueous AcOH provided the desired tri-*O*-acetyl-D-glucal, which was used in a Ferrier reaction with methanol/Dowex H^+ to give (α-methyl-2,3-dideoxy-4,6-di-*O*-acetyl-D-glucopyranoside. Purification by silica gel chromatography; followed by treatment of the anhydrous material with OsO_4 in pyridine afforded the desired diacetylated α-methyl mannoside. Flash chromatography followed by deacetylation with NaOMe/MeOH provided AMM in an 20% overall yield.

NMR spectroscopy

Two samples were prepared: (1) a 1 mM MBP-A, 1 mM AMM, 10 mM NaCl, 1 mM Tris-Cl, 25 mM $CaCl_2$ (pH 7) in 500 µL of 5% (w/v) bicelle (DMPC/DHPC 3:1 molar ratio) solution in ^2H_2O, and (2) 1 mM AMM, 10 mM NaCl, 1 mM Tris-Cl (pH 7) solution prepared in ^2H_2O. All [illegible] prepared on [illegible] equipped with a [illegible] gradients. In all cases, bicelle alignment was monitored by 1D ^2H NMR quadrapolar splittings as a function of temperature with maximal alignment observed at 39 °C. Spectra were acquired using normal hetero-nuclear (^1H-^{13}C) single quantum coherence (HSQC) experiments modified so that ^{13}C couplings were present in the direct proton dimension. Quadrature detection in the t_1 evolution period was accomplished using gradient coherence selection. For all experiments, 1024 points per scan, 16 scans per increment, 160 t_1 increments were acquired with a direct sweep width of 3000 Hz and an indirect sweep width of 9000 Hz. Using the same acquisition parameters, another identical HSQC experiment was acquired at a temperature of 25 °C where the bicelle medium is in an isotropic solution state.

Calculations of dipolar couplings and order tensors

Dipolar couplings were calculated as the difference between the oriented couplings ($^1J_{CH} + D_{CH}$) and the isotropic couplings ($^1J_{CH}$). In each case, couplings were extracted using a Bayesian time-domain NMR parameter estimation program Xrambo, using the method described (Andrec & Prestegard, 1998). This program is available on the Internet at http://tesla.ccrc.uga.edu. Typically, a 2D HSQC data set with the desired splittings in the direct dimension was transformed and phased to yield a 1024 × 256 real matrix. Single FID slices across the width of the resonance under investigation were analyzed independently. For every slice, the resulting frequency domain data were reverse Fourier transformed to generate a 1D time domain data set as input for Xrambo. The following model was used for the data. Each component of the doublet resulting from C-H couplings was given an identical linewidth and intensity, but an independent phase to circumvent any problems resulting from the presence of phase twist anomalies or dispersive contributions. Values for shifts, linewidths, phases and intensities were estimated and entered as starting parameters which were subsequently refined by Xrambo's Metropolis Monte Carlo method. This procedure was repeated for several indirect slices of the same doublet and the resulting rmsd across various slices was used as the uncertainty in measurement. The error analysis from this procedure was used to estimate the final precision of all measured residual dipolar couplings. The measured residual dipolar couplings and associated uncertainties along with AMM input coordinates obtained from an MD simulation were then used as input to a singular value decomposition program for the determination of order tensor elements (Losonczi *et al.*, 1999). This

program is also available on the internet at http://tesla.ccrc.uga.edu.

Molecular dynamics simulation

MD simulations were performed with AMBER 4.1 (Pearlman *et al.*, 1995) employing the all-atom GLYCAM93 parameter set for oligosaccharides (Woods *et al.*, 1995) on a 12 processor SGI Origin 2000 computer. AMM was solvated in an approximately 32 Å x 32 Å x 32 Å box of 466 TIP3P water molecules and was minimized by steepest descent energy minimization using a dielectric constant of 1.0 and a cut-off value for non-bonded pair interactions of 8.0 Å. Initial atomic velocities were assigned at 5 K, and the simulation run at 300 K at 1 atm. Selected torsions were extracted with the CARNAL module of AMBER 4.1 from the trajectories to determine a closely related family of the most populated structures.

Docking studies

First approximations of AMM's position in MBP were done with MIDAS Plus (Ferrin *et al.*, 1988). Briefly, MBP with AMM modeled into the CRD were aligned with the helix parallel with the z-axis. A second AMM was matched and oriented according to the singular value decomposition analysis of the calculated dipolar couplings (a $z-10°$, $y+40°$ rotation from the crystal structure). This was then translated in x, y, and z directions while limiting rotations to the z-axis only. Solutions that maintained the three and four OH groups 2.6-2.8 Å from the Ca^{+2} responsible for binding and that minimized van der Waals interaction with the protein were deemed plausible solutions.

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